THERMOSTABLE SINGLE-BAND CYTOCHROME OXIDASE

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1. Introduction

There remains strong controversy over the energy transforming mechanism of cytochrome oxidase (EC 1.9.3.1) [1]. To understand this mechanism at the molecular level, a simple and stable oxidase is desirable. The mitochondrial cytochrome oxidase, is best characterized, containing seven subunits [2].

Some species of bacteria have been known to contain aa_3-type cytochrome as a terminal oxidase of the respiratory chain [3,4]. Properties of partially purified oxidases from *Nitrosomonas europaea* [5], *Mycobacterium phlei* [6] and *Thiobacillus novellus* [7] have been reported without a description of molecular structure.

Here we have purified a stable cytochrome oxidase from thermophilic bacterium PS3 [4], whose catalytic properties are very similar to those of the mitochondrial enzyme but whose subunit structure is simple: a single band (38 000 mol. wt) is seen in SDS–polyacrylamide gel electrophoresis. This PS3 oxidase is able to transform redox energy into chemiosmotic energy when reconstituted into liposomes.

2. Experimental

2.1. *Materials*

Equine cytochrome *c Candida krusei* cytochrome *c* were purchased from Sigma (St Louis) and Sankyo (Tokyo), respectively. Soybean P-lipids were obtained and partially purified as in [8]. A cyanine dye, 3,3'-diethylthiodicarbocyanine iodide was a product of Eastman Kodak (New York).

2.2. *Purification of cytochrome oxidase of PS3*

The membranes were treated with cholate plus deoxycholate as in [9] and washed with 4 M LiCl. The resulting residue was extracted with 4% Triton X-100. This extract was purified by chromatography in the presence of Triton X-100. DEAE-cellulose column was equilibrated with 50 mM Tris · HCl buffer (pH 8.0) containing 0.5% Triton X-100, on this column, PS3 oxidase was not absorbed. It was absorbed on a second DEAE-cellulose column equilibrated with water; the column was washed with 30 mM Tris · HCl buffer (pH 8.0) containing 0.5% Triton X-100, and the oxidase was eluted with 60 mM Tris · HCl (pH 8.0) containing 1% Triton X-100. The combined fractions of the eluate were applied on a hydroxyapatite column equilibrated with 0.5% Triton X-100 and 1 mM P_i buffer (pH 7.0), washed with 5 mM P_i containing 0.5% Triton X-100, and eluted by raising P_i to 50 mM. The greenish-brown eluate was mixed with sodium cholate (1.5% final conc.) and precipitated by adding 220 mg/ml of solid ammonium sulfate while stirring. The precipitate was collected and suspended in 5 mM Tricine · NaOH (pH 8.0). This was used as PS3 cytochrome oxidase.

2.3. *Methods*

Absorption change of the cyanine dye was used for measuring the membrane potential [10]. Light of the wavelength pair (630–670 nm) was applied to a cuvette of 1 cm lightpath in a Hitachi dual wavelength spectrophotometer, model 356, at 38°C. The concentrations of cytochromes aa_3 and *c* were determined

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spectrophotometrically using molar extinction coefficient increments of 16.5×10^3 M^{-1} .cm^{-1} (604–630 nm, reduced state), and 16×10^3 M^{-1} .cm^{-1} (550–528 nm, reduced state). Protein, P-lipid and Triton X-100 contents were assayed as in [11].

3. Results and discussion

Cytochrome oxidase activity and cytochrome aa_3 content of the original membranes and the purified oxidase are shown in table 1. Purification was 35 times for cytochrome aa_3 content and 17 times for the activity. When ascorbate with phenazine methosulfate was used as a substrate, a similar enrichment of the activity was observed.

Figure 1 shows an electrophoretic pattern of PS3 oxidase on SDS–polyacrylamide gel. A single band was observed at the position of 38 000 mol. wt, either with or without succinylation of the protein.

Figure 2 shows the reduced minus oxidized difference spectrum of PS3 oxidase. Apparently the absorption peaks of 445 and 604 nm were indicative of cytochrome aa_3 and 418, 521 and 550 nm, of cyto-

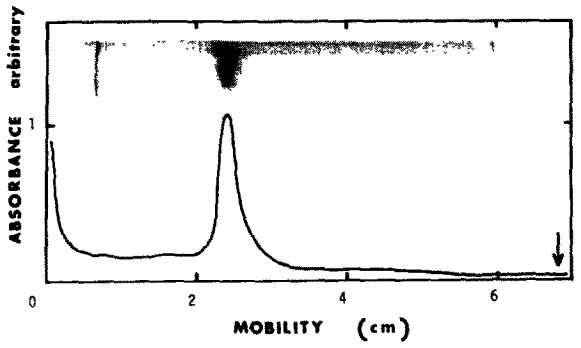

Fig.1. Electrophoretic pattern of purified PS3 cytochrome oxidase. SDS–gel electrophoresis was carried out in 7.5% gel containing 8 M urea by essentially the method in [15]. PS3 oxidase (50 μg) was first succinylated with succinic anhydride (1 mg) in 0.1 M Na_2CO_3–$NaHCO_3$ buffer (pH 8.0) for 30 min at 25°C and an aliquot (~5 μg protein) was applied on the gel after denaturation with a mixture containing 8 M urea, 4% SDS and 5% mercaptoethanol.

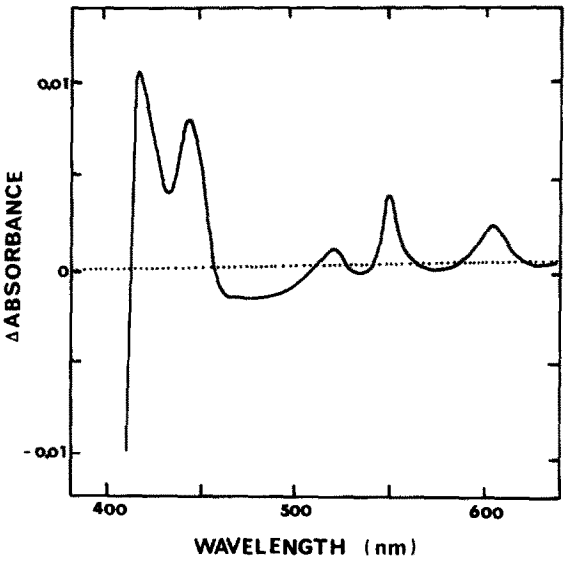

Fig.2. Difference spectra of PS3 oxidase. Both sample and reference cuvettes (10 mm lightpath) contained 82 μg protein of PS3 oxidase in 0.7 ml 50 mM Tris · HCl buffer (pH 7.4) 0.1% Triton X-100. (- - - -) Base line (oxidized minus oxidized); (——) dithionite reduced minus oxidized.

Table 1
Cytochrome *c* oxidase activity of membrane fraction and purified oxidase of PS3

Fraction	Total prot. (mg)	Spec. act. (k/mg prot.)	Total act.	Cytochrome aa_3 content (nmol/mg prot.)	Total cyt. aa_3 (nmol)
Membrane fraction	1150	0.56	644	0.32	276
Purified oxidase	2.4	9.7	23	11.4	27

Cytochrome oxidase activity was assayed by following the A_{549} of yeast ferrocytochrome *c* at 15 μM in 1 ml 50 mM P_i buffer (pH 6.1) containing 50 μg sonicated P-lipids at 25°C

Table 2
Composition of PS3 cytochrome oxidase

Component	Amount per mg protein	
	Prep. 1	Prep. 2
Cytochrome aa_3	13.5	11.3 nmol
Cytochrome *c*	13.9	11.0 nmol
Total copper	22.6	19.0 nmol
Intrinsic copper	13.6	7.8 nmol
Phospholipids	0.06	0.10 mg
Triton X-100	0.99	0.08 mg

chrome *c*. Composition of two typical preparations are summarized in table 2. Besides cytochromes PS3 oxidase contains copper like mitochondrial oxidase [12]. Intrinsic copper, which is reactive with bathocuproine sulfate in the presence of dodecyl sulfate [13], was found in a similar concentration as in mitochondrial cytochrome oxidase. Considering the low heme to protein ratio, if heme was not detached during purification, it is likely that two 38 000 mol. wt proteins contain 1 heme *a*, 1 heme *c* and 1 Cu. The relationship between heme *a*, heme *c* and copper, or between two peptides remains to be elucidated. In any case, the subunit structure of PS3 oxidase is quite simple in comparison to mammalian and yeast cytochrome oxidases which have been reported to be composed of seven kinds of subunits [2,14]. PS3 oxidase was able to oxidize yeast cytochrome *c* rapidly, when P-lipids or nonionic detergents such as Tween-20 were added. In contrast, equine cytochrome *c* was only oxidized about 5 times more slowly than yeast cytochrome *c*. Hydrophobic cytochrome c_{551} purified partially from PS3 membranes was oxidized as rapidly as yeast cytochrome *c*. The general catalytic properties of PS3 oxidase were very similar to mitochondrial oxidase: cytochrome *c* oxidase activity was inhibited by a high ionic strength or inhibitors such as 0.1 mM cyanide (96%), 1 mM azide (60%) and 3 mM hydroxylamine (80%).

Stability of PS3 oxidase was excellent: PS3 oxidase shows 100% activity after preincubation at 63°C and 50% of the activity after incubation at 70°C for 10 min. PS3 oxidase was stable against denaturating reagents such as 7 M urea, 5 M LiCl and 40% ethanol.

PS3 oxidase was able to generate a membrane potential when reconstituted into vesicles. Figure 3

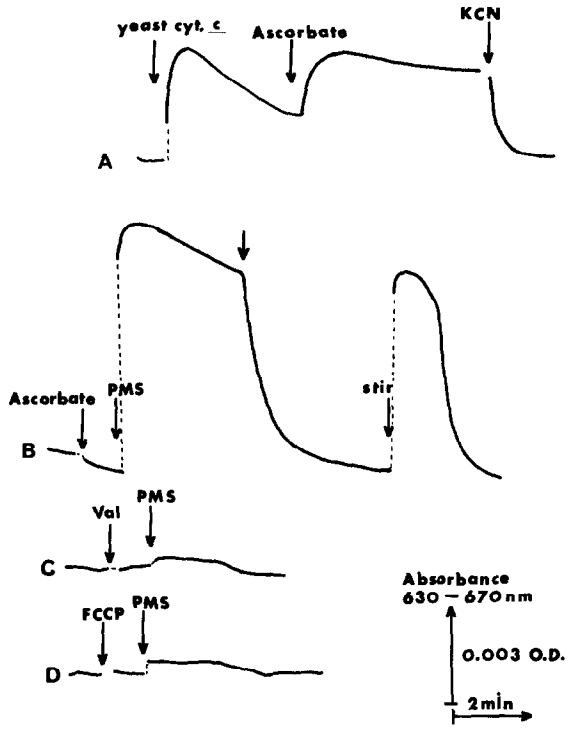

Fig.3. Formation of membrane potential by vesicles reconstituted from PS3 oxidase. Vesicles were prepared from 60 μg protein of PS3 oxidase and soybean P-lipids in 1 ml 20 mM P_i buffer (pH 6.4) by the freeze-thaw method [16]. Vesicles (50 μl) were suspended in 50 mM Tris · H_2SO_4 (pH 7.6) containing 20 mM Na_2SO_4, 2 mM $MgSO_4$ and 13 μM cyanine dye, with the exception of (C) which contained additionally 13 mM KCl. Additions were as follows: yeast cytochrome *c*, 50 μl 1.5 mM; sodium ascorbate, 2 mg; KCN, 20 μl, 0.1 M; PMS (phenazine methosulfate), 1 μl, 10 mM; Val (valinomycin), 5 μl, 0.1 mg/ml; FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), 10 μl, 0.1 mg/ml.

shows the change of $\Delta A_{630-670}$ due to a cyanine dye, which is known to be proportional to an electrical potential formed across membranes [10]. Addition of yeast cytochrome *c* resulted in a rapid change of ΔA, which gradually decreased as oxidation of cytochrome *c* proceeded (A). Addition of ascorbate stimulated the absorption difference again via ferrocytochrome *c*. Inhibition of oxidation by cyanide abolished the absorption change due to membrane potential formation. Ascorbate was not oxidized by PS3 oxidase and thus the absorption difference remained constant, and addition of phenazine methosulfate resulted in a

rapid increase of the absorbance. After oxygen in the reaction medium was exhausted (↓), this increment gradually disappeared and reappeared upon stirring with introducing oxygen (B). This process was repeated several times. In contrast, only little, if any absorption change was observed in the presence of valinomycin (C) or FCCP (D).

The present investigation shows that subunit composition of PS3 oxidase is very simple and still possesses activities of electron transfer and energy transformation. More detailed properties of PS3 oxidase are now under investigation and will help to elucidate the membrane energetics of respiratory components.

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